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Notch4 expression in the mouse mammary gland is associated with mammary gland tumors. Previously we demonstrated that a member of nuclear hormone receptor family interacts with Notch1. One functional consequence of this interaction is an anti-apoptotic effect in cells expressing activated Notch1. We hypothesized that Notch4, like Notch1, might interact with other members of the nuclear hormone receptor family in mammary gland epithelium and this may prevent the normal apoptotic cell death that occurs during remodeling in the post-lactating breast. The aims in this proposal are designed to test this hypothesis. Results are discussed suggesting that Notch4 may regulate progesterone receptor signaling.

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B A Osborne 6/25/99

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Table of Contents

INTRODUCTION:

This proposal is designed to explore the premise that Notch4 signaling may regulate apoptosis in the mammary gland. Three specific aims were proposed to address this premise. The aims are 1) to determine if nuclear hormone receptors interact with Notch; 2) to determine the functional role of Notch and nuclear hormone receptors in tumor formation; 3) to elucidate the signaling pathways influenced by potential interaction between Notch and nuclear hormone receptors. During the past year, year 1 of the proposal, we have made significant progress toward the completion of aim 1 and have begun aims 2 and 3.

BODY:

The following paragraphs contain a summary of the work accomplishments completed or in progress for the grant proposal entitled "The role of Notch in regulating apoptosis in the mammary gland". The first aim of this proposal was to determine potential protein/protein interactions between members of the Notch family and members of the nuclear hormone receptor family. In this aim we proposed to do a yeast two-hybrid screen of Notch with various hormone receptors, to look for these interactions in vivo in mammary epithelial cells, and to determine if these interactions might be physiologically relevant to involution by analysis of the expression of these receptors. To address this aim, we chose to use a mammalian two-hybrid screen instead of yeast since interaction in mammalian cells is more relevant to the questions we posed. When we first submitted this proposal, mammalian two-hybrid assays were in their infancy and vectors were not commercially available so yeast two-hybrid assays were proposed. We cloned Notch4 into the Clontech mammalian two-hybrid "prey" vector and Nur77, progesterone receptor A and B were cloned into the "bait" vector. We are still in the process of cloning the glucocorticoid receptor, the estrogen receptor, the vitamin D3 receptor, and all of the RAR and RXR family members. We have demonstrated the mammalian two-hybrid assay works in our hands and have found no interaction between the progesterone receptors and Notch and a possible interaction between Nur77 and Notch in mammalian cells. The latter possible interaction will require more experiments because the potential interaction is quite weak.

To look for possible indirect interactions or effects of Notch activity on steroid receptor signaling, we cloned Notch1 and Notch4 into a Tet-inducible vector for stable transfectants and into the expression vector, pCDNA3, for high level expression in transient and stable transfections. We are in the process of isolating cell lines that express different levels of Notch1 and Notch4. These constructs are the first reagents we will need to address the tasks suggested in Aim 3, to analyze the signaling pathways influenced by Notch on steroid hormone receptor signaling. We have conducted transient transfections of Notch1 and 4 with reporter constructs containing progesterone receptor elements, estrogen receptor elements, and retinoic acid receptor elements to analyze these effects. We have found that Notch proteins can repress progesterone signaling but not signals induced by the addition of all-trans retinoic acid. Since there was no direct interaction between Notch4 and either progesterone receptor isoform, we

are investigating the role of various signaling pathways in the repression of progesterone receptor transactivation by the use of dominant negative proteins and inhibitors. Using mutant forms of Notch we will also be able to determine if the repression maps to the RBP-Jk/CBF-1 domain or another domain of Notch. We have a reporter plasmid for RBP-Jk activity and future plans include determining if steroid receptor signaling might influence this aspect of the Notch signal, however, given that this does not appear to be a direct interaction with Notch, we think it is unlikely to have an effect.

In Aim 2, we proposed to look at the role of Notch in tumorigenesis. In this part of the project we hope to be able to conduct *in vivo* experiments by taking advantage of the ability to retrovirally infect purified mammary epithelial cells or cell lines such as comma D and place the cells back in a cleared fat pad. Normal cells will form a typical branching morphology and go on to differentiate properly while Notch4 (Int3) expressing cells will have a block in development and form tumors. We hope that our biochemical studies of the signaling pathways leading to repression of certain steroid receptor responses in mammary epithelial cell lines will elucidate a pathway to the repression. In this case we will then be able to interrupt this pathway by expression of a dominant negative or constitutively active protein and hopefully revert the phenotype in vivo. At that point we will be able to analyze the importance of steroid repression in Notch4 induced tumors, differentiation, and death. We have an epitope-tagged form of Notch1 and 4 in a retroviral vector for this purpose, but we are not ready to proceed to the next step yet.

Although not part of the defined goals of the proposal, to address the issues in the three specific aims, it was necessary to establish cell culture conditions for mammary gland epithelial cells. Additionally, it was necessary to establish conditions to induce differentiation and induction of apoptosis in these cells. During the past few months we have been able to both differentiate and induce apoptosis in HC11 cells, a cell line derived from comma D cells. We also have made a peptide from the C-terminal of the Notch4 protein and generated a rabbit polyclonal antibody against that peptide. Western blot analysis established that the antibody reacted with cells expressing Notch4.

KEY RESEARCH ACCOMPLISHMENTS

- Mammalian two-hybrid system functional
- Notch cloned into mammalian two-hybrid vectors, Tet-inducible vectors, retroviral vectors, and a good CMV-based vector for high level stable and transient expression.
- Reporter assays for steroid receptor signaling and Notch signaling set up and working.
- Antibodies specific to mouse Notch4 have been generated.

REPORTABLE OUTCOMES

- HC11 cell lines stably expressing Notch4
- Polyclonal rabbit antibodies made against the C-terminus of mouse Notch4.

CONCLUSIONS

We have assembled and constructed most of the tools necessary to conduct the proposed studies. We have established the mammalian two-hybrid assay in our laboratory. We have determined conditions for the growth, differentiation and induction of apoptosis in HC11 cells, a derivative of comma D cells. We have found that Notch4 and the progesterone receptor do not physically interact in mammalian two-hybrid assays however Notch4 and Nur77 may interact. It remains to be determined if other nuclear hormone receptors interact with Notch4. Lastly, we have made the potentially important discovery that Notch expression represses the function of the progesterone receptor. This may have important implications in situations where Notch4 is overexpressed. Since Notch4 overexpresssion in detected in mammary tumors (L. Miele, personal communication), the influence of Notch4 on progesterone receptor function may prove to be quite important in our understanding of mammary gland tumorigenesis.